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THE EFFECT OF PROPYLTHIOURACIL ON THE OXYGEN  
CONSUMPTION AND RADIOIODINE METABOLISM  
OF THE FROG, RANA PIPIENS, IN WINTER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES  
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE  
OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

BY

HERMAN MATTHEW MEISNER

EDMONTON, ALBERTA

APRIL, 1961





## ABSTRACT

This study was undertaken to evaluate the physiological importance of the thyroid gland in the adult frog, Rana pipiens, in winter. Male frogs, weighing between 40-55 grams, were kept in running water and fed liver once a week. The thyroid gland was chemically inhibited by oral administration of 1.5 mg. of propylthiouracil three times a week. Oxygen consumption was measured weekly from December 21 to February 23 with volumetric respirometers at a temperature of  $16 \pm 0.1^{\circ}\text{C}$ .

There were never any significant differences between the metabolic rates of the propylthiouracil inhibited frogs and the controls over the entire experimental period. Propylthiouracil effectively blocked the thyroid radioiodine uptake and blood conversion ratio, but after nine weeks, thyroid iodine stores were only reduced by one-half. This low secretion rate together with the fact that propylthiouracil did not alter metabolic rate suggests that the frog thyroid is of no calorogenic importance during the winter.

The seasonal thyroid activity pattern was found to be characterized by a regression in the iodine concentrating capacity of the thyroid gland and blood from August to December, and reactivation towards the end of February.



The appearance of monoiodotyrosine and diiodotyrosine in the circulation of Rana pipiens indicates that thyroidal deiodinase is unable to reduce iodotyrosines to iodide. It is postulated that there is an association between the presence of iodotyrosines in the circulation and the reduced thyroid function of poikilotherms.



## ACKNOWLEDGEMENTS

It is a pleasure to acknowledge my indebtedness to my advisor, Dr. C. P. Hickman, Jr., for suggesting the thesis topic and for his helpful advice and criticism in all phases of the experiment. I would like to express my thanks to Dr. G. O. Mackie and to Dr. G. MacLachlan for their valuable suggestions and help in the histological and radioautographic work. I also wish to thank Mr. D. H. Sheppard for his assistance in the chemical iodine analyses.





## TABLE OF CONTENTS

	<u>Page</u>
Introduction -----	1
Methods and Materials	
Care and Feeding of Frogs -----	4
Apparatus -----	6
Measurement of Oxygen Consumption -----	7
Conversion Ratio Measurement -----	8
Chemical Iodine Measurement -----	9
Radioiodine Uptake Measurement -----	10
Radioautography -----	11
Results	
Effect of Feeding -----	12
Effect of Light and Time in Respirometer -----	12
Diurnal Variation in Metabolic Rate -----	14
Required Level of Propylthiouracil -----	16
Effect of Lactose on Oxygen Consumption -----	17
Effect of Propylthiouracil on Oxygen Consumption ---	18
Conversion Ratio Measurements -----	20
Chemical Iodine Measurements -----	21
Uptake of Radioiodine -----	24
Radioautographic Studies -----	24
Discussion	
Factors Affecting Metabolic Rate -----	27
Thyroid Iodine Metabolism	
A. Influence of Season -----	31
B. Effect of Propylthiouracil -----	34
Significance of Circulating Iodotyrosines -----	35
Conclusions and Summary -----	38
Bibliography -----	41
Appendix -----	49





## LIST OF TABLES

	<u>Page</u>
Table I. Effect of propylthiouracil on conversion ratio. -----	16
Table II. Effect of lactose on oxygen consumption. -	18
Table III. Effect of propylthiouracil on oxygen consumption. -----	50
Table IV. Effect of propylthiouracil on body weight.	51
Table V. Chemical protein-bound iodine analysis of frog blood and thyroid glands. -----	25



## LIST OF FIGURES AND PLATES

	<u>Page</u>
Fig. 1. Effect of liver feeding on oxygen consumption.--	13
Fig. 2. Effect of light and darkness on oxygen consumption. -----	13
Fig. 3. Daily variation in oxygen consumption. -----	15
Fig. 4. Effect of propylthiouracil on oxygen consumption. -----	19
Fig. 5. The conversion ratio as influenced by propylthiouracil and season. -----	22
Fig. 6. Radioiodine uptake of normal and propylthiouracil inhibited winter frogs. -----	25
Plate 1. Volumetric respirometer - partially submerged.-	52
Plate 2. Volumetric respirometer - measuring position.--	52
Plate 3. Radioautograph of normal frog thyroid. -----	53
Plate 4. Radioautograph of propylthiouracil inhibited thyroid gland. -----	53





## INTRODUCTION

While a vast amount of knowledge has been accumulated on the thyroid gland of homiothermic animals, relatively little is known about the thyroid of cold blooded vertebrates, particularly the Amphibia. The existence of the thyroid gland in amphibians had been recognized since the middle of the nineteenth century, but nothing more became known about it until Cameron in 1914 demonstrated the presence of iodine in the gland of Rana pipiens by means of a crude chemical analysis. That same year Gudernatsch began a series of experiments which eventually proved that the thyroid was responsible for the onset and continuance of metamorphosis in the tadpole.

However, thyroid function in the adult Amphibian remained somewhat obscure until the last two decades. There is now good evidence that it is responsible for molting cycles. Extirpation of the gland results in cessation of molting while injection of thyroid hormone will recommence the cycle (Adams and Richards, 1929; Warren, 1940). Warren (op. cit.) drew attention to the importance of the gland in metabolism by showing that injection or feeding of thyroid extracts would cause an increase in oxygen consumption. Employing the newly discovered radioisotope of iodine,  $I^{131}$ , Matthews (1950) demonstrated





that the gland accumulates a small, but measurable amount of iodine, and that this uptake could be blocked by administration of thiouracil. Shellabarger and Brown (1959) have recently shown that iodine synthesis and hormone production in the amphibian thyroid gland is similar to other vertebrates.

The importance of the gland at different seasons of the year has not been well established. The endocrine glands of poikilotherms in general undergo a seasonal cycle of activity. The period of highest activity seems to occur in the spring and summer, followed by a gradual regression until a low level is reached during the winter months. During the winter, the thyroid gland of the frog is at a period of colloid storage rather than colloid release (Joel, et al., 1949).  $I^{131}$  uptake studies with Amphiuma have indicated that the rate of release of radiothyroxine from the thyroid gland is very slow in the winter (Berg, in Gorbman, 1959).

Inhibition of the thyroid gland of mammals with goitrogens depresses thyroid function and lowers the basal metabolic rate (Astwood, 1943, 1945; Mackenzie, 1943). Similarly, propylthiouracil has been shown to lower the basal metabolic rate of the ~~summer~~ frog (Calhoun, 1955) in the summer.



In view of the seasonal variation of the frog thyroid gland, a study of the effect of a goitrogen on thyroid activity in the winter frog<sup>1</sup> would be valuable. This experiment was therefore designed to measure the effect of propylthiouracil on the oxygen consumption and radioiodine metabolism of the adult frog, Rana pipiens, in the winter.

<sup>1</sup> The terms "winter frog" and "summer frog" are used in the account of the experiment to denote the frog, Rana pipiens, as studied during the winter (December 21 to February 23) and summer months (July - August).



## METHODS AND MATERIALS

### Care and Feeding of Frogs.

Adult, male Rana pipiens weighing 40-55 grams were obtained from the Steinhilber Company of Oshkosh, Wisconsin. Upon arrival they were weighed and numbered by cutting off the terminal digits of front toes in various combinations. These toes were then swabbed with coal tar to prevent infection.

The frogs were kept in a galvanized tank measuring 28 inches high, 2 feet wide, 7 feet long, and covered with a wire screen. A continuous stream of dechlorinated water flowed into a shallow metal pool at the raised end of the tank. The temperature of this water remained between 13-15°C throughout the experiment. A fine mesh wire screen divided the tank longitudinally into two compartments, separating experimental animals from the stocks. A third section, located near the drain at the lowest end of the tank, was used to isolate frogs injected with radioiodine. The tank was disinfected with soap and water once a week to prevent infection.

Despite efforts to reduce disease in the colony, some difficulty was experienced with redleg, a skin infection caused by the bacterium Pseudomonas hydrophilis.





The remedy finally adopted was to bathe the animals every other day in a weak aqueous solution of the germicide, Nitrofurazone (VioBin (Canada) Ltd., Vancouver, British Columbia). This was effective and kept mortality at a negligible level.

The frogs were fed once a week on a diet of raw beef liver and finely ground rabbit pellets, the latter added to provide an adequate intake of vitamins and minerals. Liver was cut into one gram strips and coated with a wet paste of the rabbit food. Frogs were then force-fed by opening the mouth with forceps and placing the liver behind the tongue. To be certain there was no regurgitation of food, the frogs were held in individual jars for one-half hour before being returned to the tank. Chemical analysis showed that the weekly intake of liver contained about 4.6 micrograms iodine per frog.

Chemical inhibition of the thyroid gland was accomplished by the administration of propylthiouracil (PTU) three times a week for the duration of the experiment. Bradley (1951) has shown that one injection of thiourea effectively blocked radioiodine ( $I^{131}$ ) uptake for three days in Rana pipiens. PTU was given orally mixed with an inert medium (lactose) and enclosed in a #5 gelatin capsule (Eli Lilly and Company, Indianapolis,





Indiana). The capsule was placed behind the tongue as in liver feeding. The gelatin capsule was completely dissolved in the stomach within six hours after feeding. This method proved more satisfactory than the conventional method of multiple injections of an insoluble suspension of PTU into the lymph sac. A precise amount of PTU could be administered with no injury to the skin.

### Apparatus

Oxygen consumption was measured by means of a volumetric respirometer with compensating chamber similar to the type described by Bliss (1953). Three such respirometers, made of lucite and fitted with stainless steel handles, were constructed. The animal chamber was a lucite cylinder measuring 5 inches in diameter and 4 inches high, with a total volume of 1800 cc. A four pound lead weight added to the bottom of the apparatus counteracted the bouyancy of the vessel in water. A 5 inch diameter rubber "O" ring fitted into an indentation under the lid provided an airtight seal between the lid and the top of the animal chamber.

Internal temperature was measured by a thermometer fitted into an offset tapered hole in the cover. KOH for carbon dioxide absorption was added through another



hole into a small lucite dish suspended from the underside of the cover. To prevent the experimental animal from accidentally contacting the KOH, a thin perforated plastic sheet was cemented to the top of the dish.

The manometer was partially filled with Brody's solution (for composition, see Umbreit, 1959). Parallel horizontal lines, 3/16 inch apart, were etched on the sides of the manometer to facilitate accurate reading of changes in the manometer fluid level. A 10 cc syringe was mounted on the respirometer cover, and filled with therapeutic oxygen to replace that consumed by the animal.

#### Measurement of Oxygen Consumption

Oxygen consumption measurements of control and PTU-fed frogs were carried out over a nine-week period from December 21 to February 23. All frogs were measured once a week and fed liver immediately after each trial. The following method is based on a series of preliminary experiments to determine the optimal conditions for measuring the basal metabolic rate (see RESULTS).

Approximately 20 hours prior to the actual measurement, the frogs were placed in totally darkened respirometers containing 5 cc of water to simulate natural conditions.





The compensating chamber contained a few cc of water to assure equal water vapor pressure in both chambers. The animal chamber was closed after placing 10 cc of a 10% KOH solution in a lucite dish at the top of the chamber. All three respirometers were partially submerged overnight in a fiberglass-lined constant temperature tank at  $16 \pm 0.1^{\circ}\text{C}$ . (see Plate 1, APPENDIX), and air was slowly circulated through the animal chamber. The following morning the respirometers were completely submerged (see Plate 2, APPENDIX) and allowed to equilibrate two or three hours to the temperature of the water bath. They were sealed off from the atmosphere at 12 noon, and oxygen consumption was measured for a four hour period. Results were expressed as  $\text{mm}^3/\text{gram}$  of wet weight/hour. After each trial, the frogs were removed and weighed to the nearest 0.1 gram after the urine had been expressed. It was found that frogs weighed on the average 2 grams more before being placed in the respirometers than afterwards. The respirometers were washed with soap and water after every trial.

#### Conversion Ratio Measurement

The conversion ratio was determined for normal and PTU inhibited frogs. Ten microcuries of  $\text{I}^{131}$  in 0.1 cc





of amphibian saline was injected intraperitoneally into each frog with a 26-gauge needle. The muscle layers covering the abdomen prevented leakage of  $I^{131}$  from the wound. From the eighth to the fourteenth day after injection, frogs were anesthetized with ether and about 1 cc of blood was terminally withdrawn from the left aortic arch. The plasma was separated, precipitated with trichloroacetic acid, and radioactivity counted with an end probe scintillation counter (Hickman, 1961). Conversion ratios were corrected for background and expressed as percentage of protein bound iodine (PBI) by the following formula:

$$\frac{PBI^{131}}{PBI^{131} + \text{Inorganic } I^{131}} \times 100$$

#### Chemical Iodine Measurement

Plasma and thyroid iodine were measured chemically by the Barker alkaline incineration method (Barker and Humphrey, 1951) following modifications used in this laboratory by C. P. Hickman, Jr., University of Alberta. Plasma from three frogs was pooled and analyzed for protein bound iodine, inorganic iodine, and butanol extractable iodine (BEI). (BEI contains only thyroxine and triiodothyronine, while PBI contains these two and other iodinated amino acids as well, presumably



monoiodotyrosine and diiodotyrosine.) Thyroid glands were removed along with a small portion of the hyoid apparatus and similarly analyzed. This was necessary because of the close association of the thyroid gland and hyoid apparatus in the frog. Preliminary  $I^{131}$  uptake and radioautographic studies indicated that the hyoid contained negligible amounts of inorganic iodine and no PBI. Blood and thyroid iodine values were expressed as micrograms percent and total micrograms, respectively.

#### Radioiodine Uptake Measurement

Radioiodine uptake studies were performed on the thyroid gland of normal and PTU inhibited frogs. Each frog was injected intraperitoneally with 10 microcuries of  $I^{131}$ , and the thyroids removed at daily intervals. Most measurements were done from the eighth to the twelfth day after injection. The thyroids were held at a uniform height above the end probe scintillation counter, and radioactivity counted. Results were expressed as percent of original dose corrected for radioactive decay.

To determine whether the cartilage left attached to the thyroid contained significant amounts of  $I^{131}$ , pieces were cut out and the radioactivity counted. The only radioactive sections were those containing the





thyroid glands. Based on this,  $I^{131}$  uptakes were determined on thyroids containing the adhering piece of hyoid cartilage.

#### Radioautography

Radioautography was employed to study the accumulation of  $I^{131}$  by the thyroid gland and hyoid apparatus. The tissues were fixed in Bouin's solution and embedded according to Griffiths and Carter (1958). Tissues were sectioned at 10 microns, mounted on slides that had previously been coated with albumin, and dried. Liquid emulsion (Ilford G5) was lightly painted over the section and allowed to dry. The emulsion was exposed two hours and developed (Fitzgerald, et al., 1953). After developing, the tissues were stained through the emulsion with <sup>n</sup>Erlich's hematoxylin and eosin (Pelc, 1956). This simple method gave excellent cellular resolution for both thyroid and adjacent cartilage cells.

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## RESULTS

Before the effect of propylthiouracil upon the oxygen consumption of frogs could be studied, it was necessary to establish optimal conditions for measuring the basal metabolic rate. A series of preliminary experiments were therefore conducted to determine the effects of different variables on the oxygen consumption.

### Effect of Feeding

It is well established that feeding increases the metabolic rate of an animal, but no data were available on the duration of this effect in the frog. To determine this, the oxygen consumption of three frogs was measured at intervals of 6, 24, 48, and 72 hours after feeding with one gram of liver.

Fig. 1 shows that oxygen consumption increased to a maximum within 6 hours, and declined to near fasting level at 72 hours after feeding. This compares closely to the results obtained with salamanders by Taylor (1939) and Belehradek and Huxley (1927).

### Effect of Light and Time in Respirometer

In these experiments, frogs were fasted two days before being placed in the respirometers. Oxygen consumption was measured during the 4-7th hour, and again during the



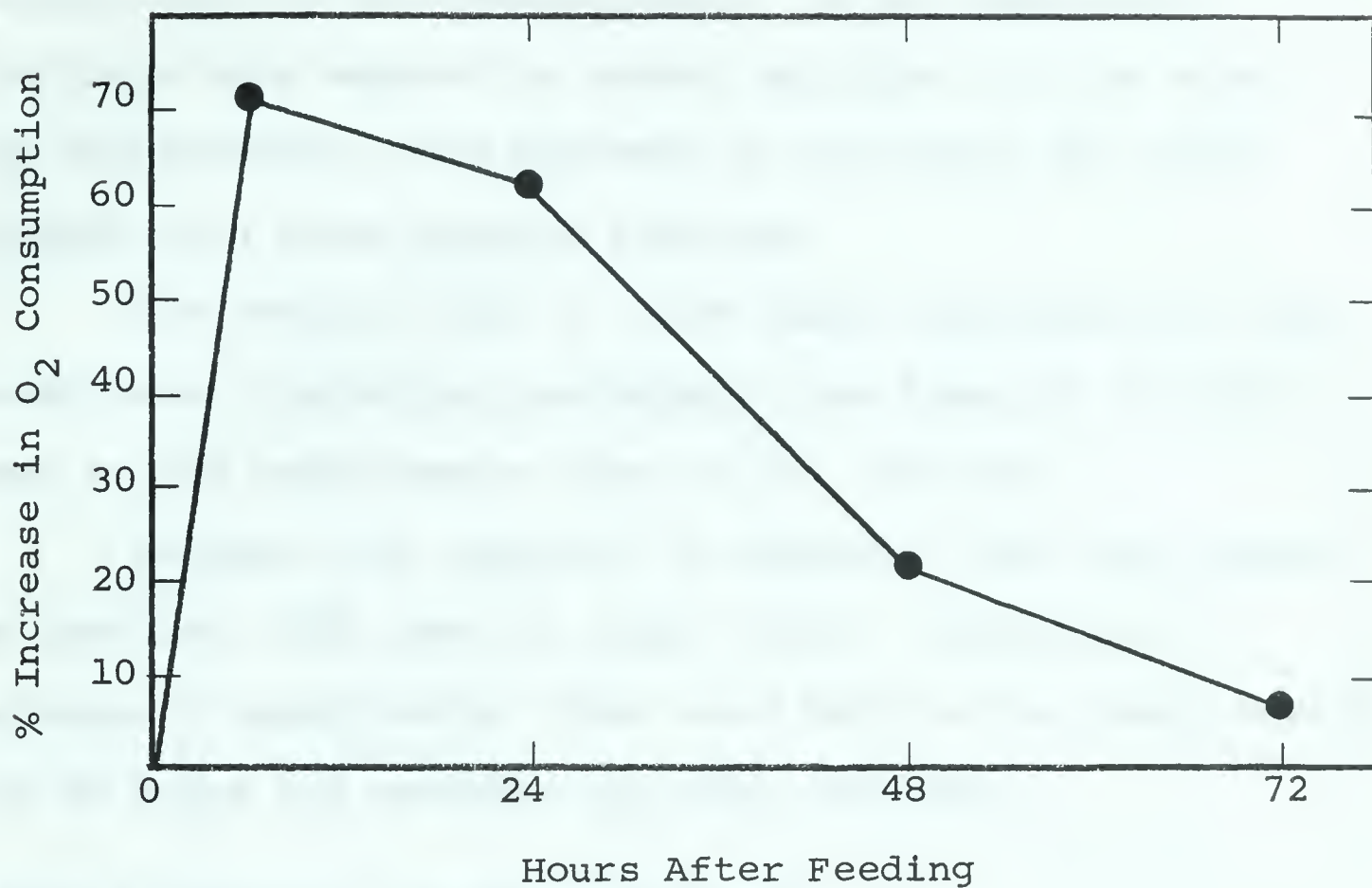


Fig. 1. Effect of liver feeding on the oxygen consumption of Rana pipiens.

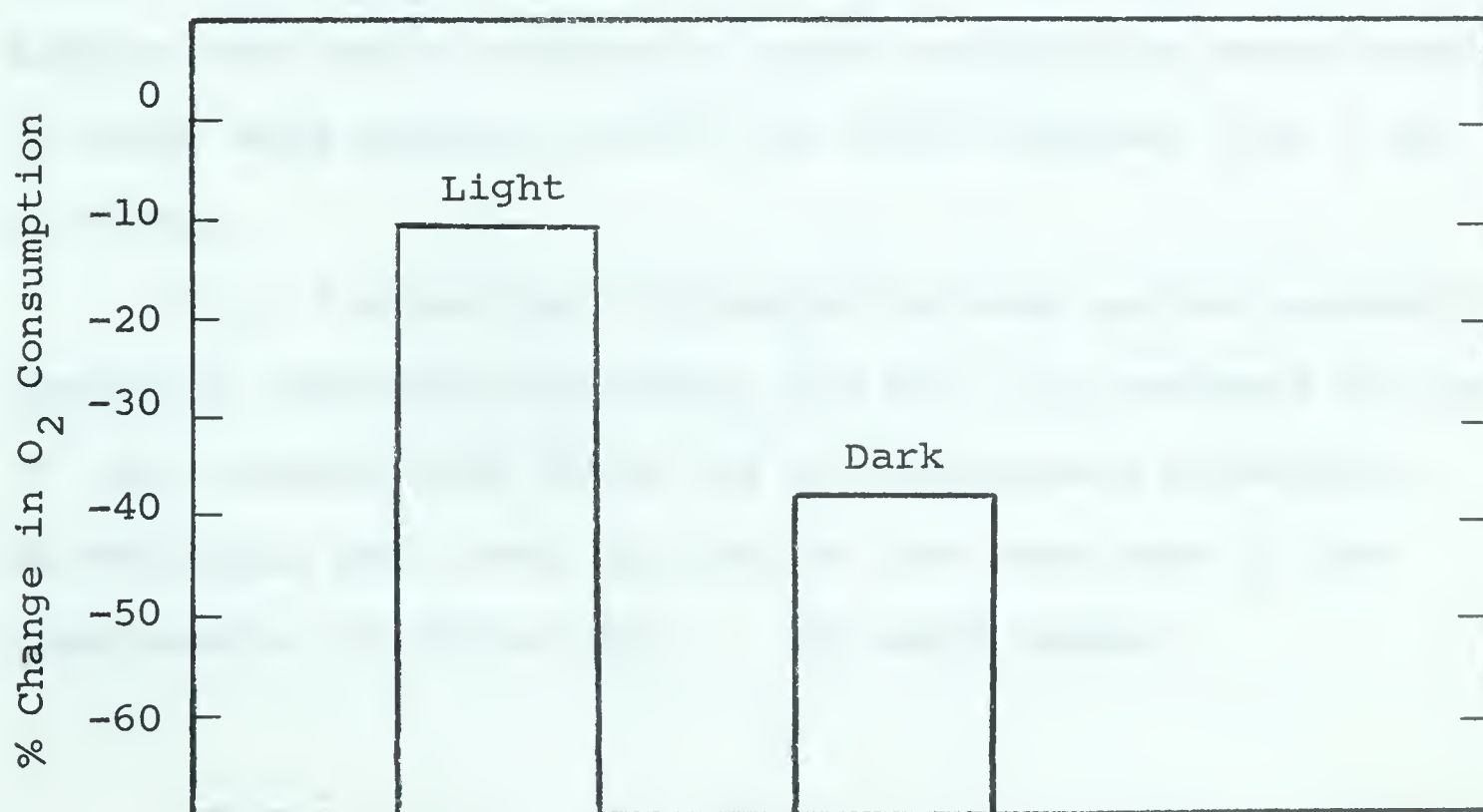


Fig. 2. Effect of light and darkness on the oxygen consumption of Rana pipiens after 24 hours in the respirometer.



21-24th hour in the respirometer. In one experiment, the frogs were exposed to normal daylight; in the other, the respirometers were darkened by enclosing the animal chamber with black plastic sheeting.

The results (Fig. 2) show that, regardless of light conditions, the oxygen consumption was lower at the 24th hour in the respirometer than at the 7th hour.

However, the decrease in metabolic rate was greater in darkness (37%) than in light (11%). Therefore, in subsequent experiments, frogs were held in the respirometers for 20 hours and measured in total darkness.

#### Diurnal Variation in Metabolic Rate

Both poikilotherms and homoiotherms show characteristic peaks of activity during a 24 hour period. To determine if frogs showed any noticeable periods of activity during the daytime that might influence oxygen consumption measurements, 12 frogs were measured hourly in total darkness from 9 AM to 5 PM.

Fig. 3 shows that although the mean oxygen consumption gradually decreased throughout the day, an analysis by the "T" test showed that there was no significant alteration in metabolic rate from the 17th to the 24th hour in the respirometer ( $0.50 > p > 0.10$ ). It would appear







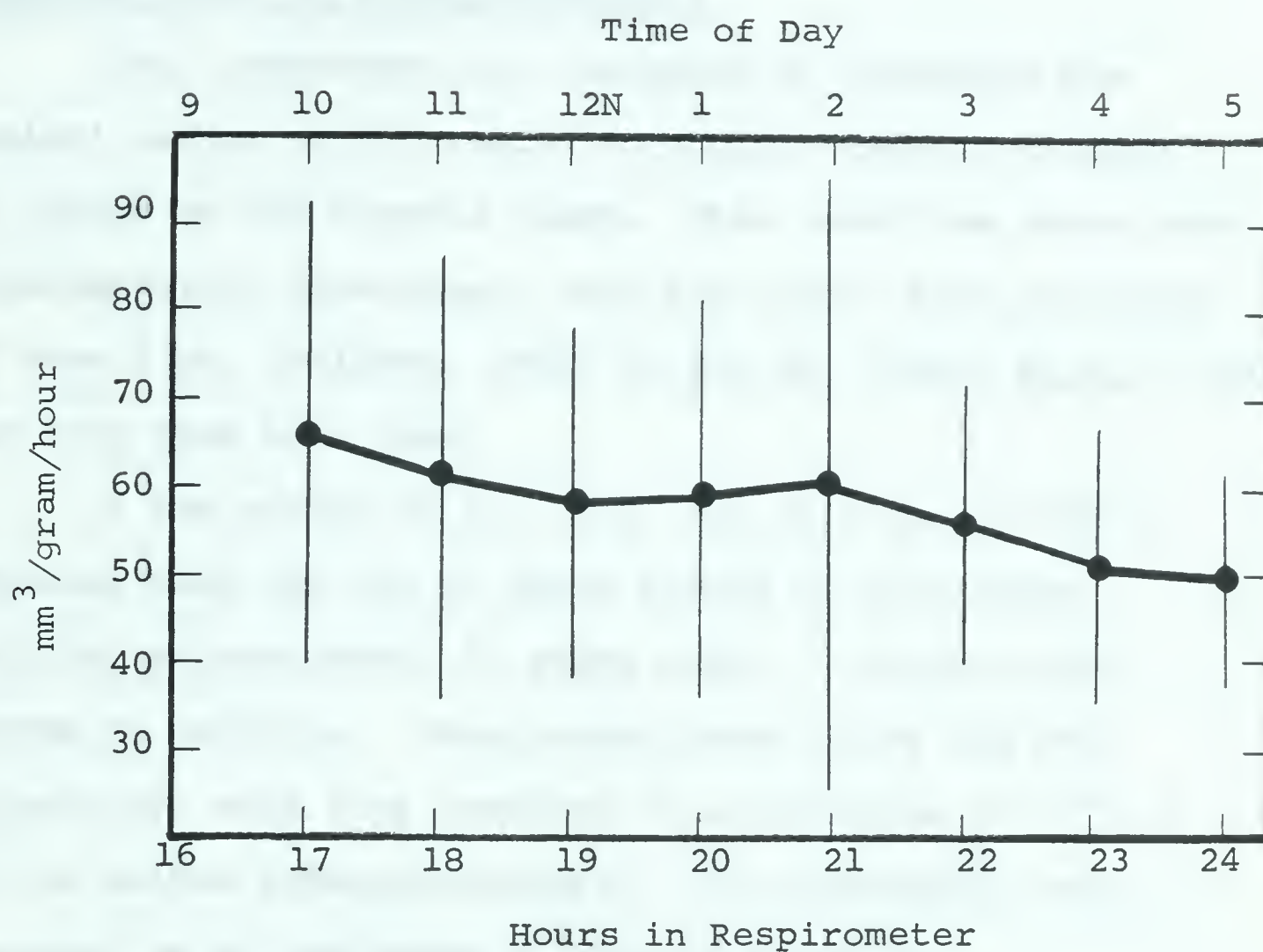


Fig. 3. Daily variation in oxygen consumption of Rana pipiens. Each point represents the mean of 12 frogs, tested under identical conditions. The vertical bars in this and the following figures represent one standard deviation.



However, because oxygen consumption was to be measured daily, a period from 12 noon to 4 PM was chosen in which to take oxygen consumption measurements.

#### Required Level of Propylthiouracil

This experiment was conducted to determine the minimal amount of PTU needed to inhibit organic binding of iodine by the thyroid gland. This level has never been experimentally determined, with the result that injection of from 1 mg. (Calhoun, 1955) to 150 mg. (Joel, et al., 1949) per frog have been used.

A log series of 0.1, 1.0, and 10.0 mg. of PTU in capsular form was fed to three groups of five frogs weighing approximately 30 grams each. A fourth group served as controls. Twenty-four hours after the PTU injections, each frog received 10 microcuries of  $I^{131}$  in 0.1 cc saline intraperitoneally. The conversion ratio was used as an indicator of thyroid activity.

Table I

Effect of Propylthiouracil on Conversion Ratio <sup>1</sup>			
<u>Control</u>	<u>0.1 mg.</u>	<u>1.0 mg.</u>	<u>10.0 mg.</u>
5.7* $\pm$ 3.0	3.8 $\pm$ 3.6	1.2 $\pm$ 0.1	1.5 $\pm$ 1.0

\* Mean  $\pm$  S.D.

<sup>1</sup> The data represent a mean of five measurements from the eighth to the twelfth day after injection of  $I^{131}$ .





Table I shows that 10 mg. and 1 mg. of PTU are equally inhibitory, while 0.1 mg. has an intermediate effect on the conversion ratio. At the level of 10 mg., the drug appeared to have severe toxic effects. All frogs showed pronounced irritability, and several died. For these reasons, 1.0 mg. PTU per 30 gram frog was chosen as the optimal amount needed to inhibit the thyroid gland.

The continual feeding of PTU over nine weeks appeared to exert a toxic effect on some of the experimental frogs. Five out of the original group of 14 PTU-fed frogs died, while all 9 of the control frogs remained alive. The PTU-fed frogs exhibited marked nervousness and sensitivity, not apparent in the control frogs. The skin of the PTU inhibited frogs was much darker than the control group at the end of the experiment. Visual observation of the thyroid glands of the PTU inhibited frogs revealed a slight hyperemia, which was not seen in the controls.

#### Effect of Lactose on Oxygen Consumption

Since 1 mg. PTU was mixed with 49 mg. of lactose for bulk and fed to the frogs in capsular form, lactose alone might influence the metabolic rate. An experiment was therefore conducted to determine the effect on the





oxygen consumption of feeding 50 mg. of lactose without PTU three times a week. Metabolic rate was measured every ten days for a full month. Results were expressed as  $\text{mm}^3/\text{gram wet weight/hour}$ .

Table II

## Effect of Lactose on Oxygen Consumption

Group	No. of Animals	Days			
		0	10	20	30
Controls	9	54.4 $\pm$ 13.9*	59.0 $\pm$ 24.5	60.5 $\pm$ 26.6	58.9 $\pm$ 16.3
Lactose Fed	6	71.4 $\pm$ 25.6	69.7 $\pm$ 29.1	53.9 $\pm$ 8.0	64.8 $\pm$ 13.5

\* Mean  $\pm$  S.D.

An analysis of Table II by a "t" test indicates that lactose did not have any significant effect on the oxygen consumption ( $p > 0.1$  in all cases).

Effect of Propylthiouracil on Oxygen Consumption

The results from the oxygen consumption measurements are summarized in Fig. 4 and in complete form in Table III (APPENDIX). The initial mean oxygen consumption is based upon the average of from 2 to 4 measurements per frog. The control group gave an initial mean oxygen consumption of  $55.3 \pm 7.1 \text{ mm}^3/\text{gram wet weight/hour}$ , while that of the PTU group was  $58.2 \pm 11.2 \text{ mm}^3/\text{gram/hour}$ .



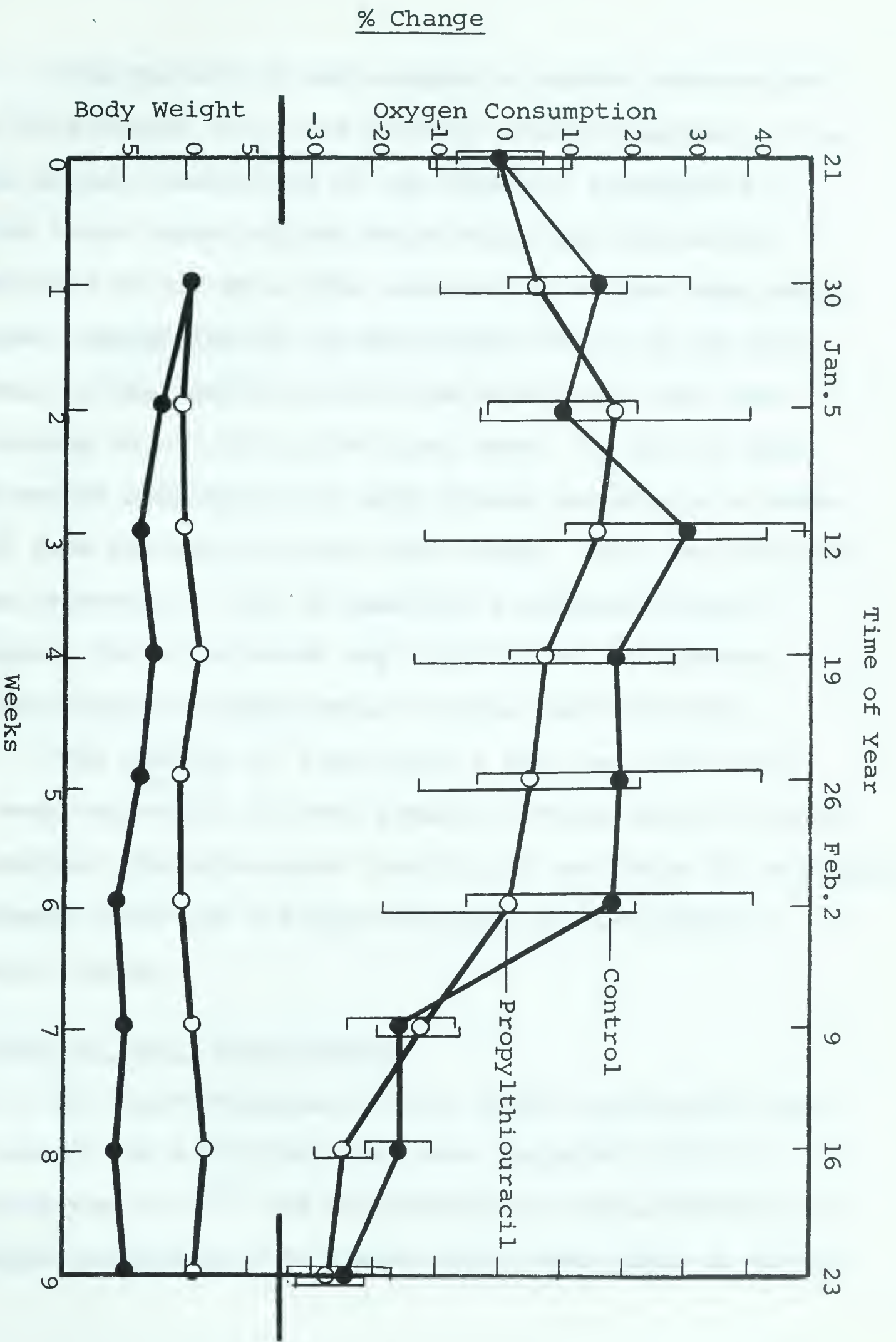


Fig. 4. The effect of propylthiouracil on oxygen consumption (top graph) and body weight (bottom graph) of Rana pipiens. Each point represents the mean of nine frogs.





The pattern of the changes in oxygen consumption for both groups coincided closely with one another. The mean oxygen consumption of the controls increased to 29.6% above normal at the third week, and thereafter decreased to -25.9% at the termination of the experiment. Oxygen consumption of the PTU frogs rose to 18.0% above normal by the second week of the experiment, and then decreased to -27.2% by the ninth week. As can be seen, the oxygen consumption of both groups registered a ~~sharp~~ drop from the sixth to the ninth week. This drop occurred about February 1, and is possibly a seasonal effect. However, there was never any significant differences in the oxygen consumption of control and PTU frogs.

The feeding of liver once a week was sufficient to keep the weight of both groups of frogs fairly constant throughout the experiment (see Fig. 4 and Table IV (APPENDIX)) although there was a slight decrease in the weight of control frogs.

#### Conversion Ratio Measurements

At the termination of the oxygen consumption work, 14 normal and 6 PTU-fed frogs were injected with 10 microcuries of  $I^{131}$  and the conversion ratios determined. Similar measurements of normal frogs were taken in August



and December. Preliminary studies showed that the plasma  $\text{PBI}^{131}$  remained about 2% until the eighth day after injection, then increased and reached a maximum by the tenth day. Fig. 5 shows the effect of season and PTU on the conversion ratio. The top line (open circles) represents a composite of normal frogs on August 1 and February 23. It shows that the plasma of these frogs contained a maximum of 7.5%  $\text{PBI}^{131}$ , while the conversion ratio of PTU-fed frogs never rose above 1.6%. The total  $\text{I}^{131}$  content per unit of plasma in PTU-fed frogs was about one-half that of the control frogs. This indicates that  $\text{I}^{131}$  was not utilized by the inhibited thyroid gland and was excreted from the body at a more rapid rate than the controls.

The middle line (closed circles) represents the conversion ratio of normal frogs taken in December. It shows that the conversion ratio was about 50% that of the normal frogs in February and August, and only slightly higher than the PTU inhibited frogs.

#### Chemical Iodine Measurements

Table V indicates that the blood PBI level of fall frogs is extremely low. The BEI/PBI ratio indicates that about one-third of the blood PBI is metabolically active





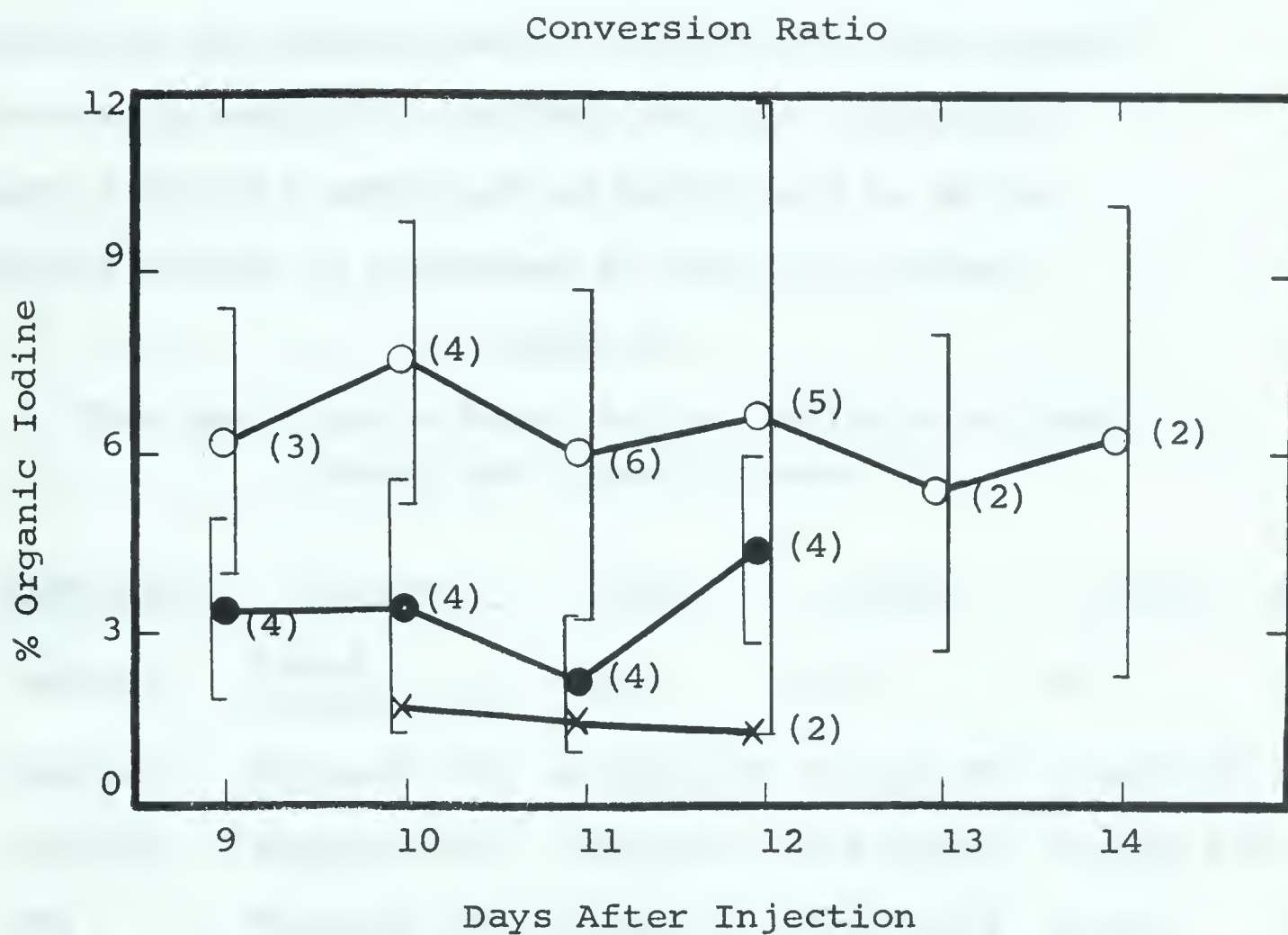


Fig. 5. The conversion ratio as influenced by propylthiouracil and season. The normal conversion ratio in August 1 and February 23 is represented by open circles, and on December 1 by closed circles. Crosses indicate effect of propylthiouracil on the conversion ratio. Numbers to the right of each point indicate number of samples.





thyroxine or triiodothyronine, while the other two-thirds consist of the lower inactive tyrosine analogs, probably monoiodotyrosine and diiodotyrosine. Analyses were also performed on the blood of PTU inhibited and normal winter frogs, but results were below reagent blank values and therefore not determinable. Since the plasma sample sizes were variable, the data are not comparable. Blood iodine is expressed as micrograms %, while thyroid iodine is expressed as total micrograms.

Table V

Chemical Protein Bound Iodine Analysis of Frog  
Blood and Thyroid Glands

<u>Date</u>	<u>Treatment</u>	<u>Tissue</u>	<u>PBI</u>	<u>BEI</u>	<u>I.I.</u>	<u>BEI/PBI (%)</u>
11/24	Control	Blood (pooled) (3)	0.24	0.07	0.71	29.2
12/1	Control	Thyroid (5)	4.30 $\pm$ 1.54*	0.42 $\pm$ 0.29	0.04 $\pm$ 0.02	8.8 $\pm$ 4.4
2/23	Control	Thyroid (3)	1.66 $\pm$ 0.01	0.07 $\pm$ 0.02	0.03 $\pm$ 0.01**	4.2 $\pm$ 1.1
2/23	PTU	Thyroid (3)	0.92 $\pm$ 0.87	0.04 $\pm$ 0.02	0.01**	4.3 $\pm$ 1.5

\* Mean  $\pm$  S.D.

\*\* Inorganic iodine not detectable in all thyroids.

() Figures in parentheses denote number of animals tested.

Thyroid I<sup>127</sup> analyses of normal frogs show that 91 and 96% of the PBI fraction, depending on the season,



consists of iodotyrosines (monoiodotyrosine and diiodotyrosine). The remaining 8.8% in December and 4.2% in February presumably is thyroxine and triiodothyronine. A seasonal effect can also be noticed for the absolute PBI and BEI levels. The total micrograms of thyroidal PBI in February fell to one third and the BEI declined to one sixth that of the December group. The percent of inorganic iodine was about 1.5% of the total, indicating a rapid oxidation of iodide to iodine. Inorganic iodine was apparently not affected by PTU or season. Propylthiouracil treatment over two months reduced both the PBI and BEI levels by 45%.

#### Uptake of Radioiodine

Fig. 6 represents a composite of thyroid  $I^{131}$  uptake experiments performed during December. The normal uptake of  $I^{131}$  by the thyroid is slow, reaching a maximum of 11.3% at the eighth day after injection of  $I^{131}$ . It remained near this level until the twelfth day, when measurements were discontinued. By comparison, the thyroid glands of PTU-fed frogs (line at lower right-hand side of Fig. 6) accumulated only 0.6% of the  $I^{131}$ .

#### Radioautography Studies

The concentration and localization of  $I^{131}$  in the





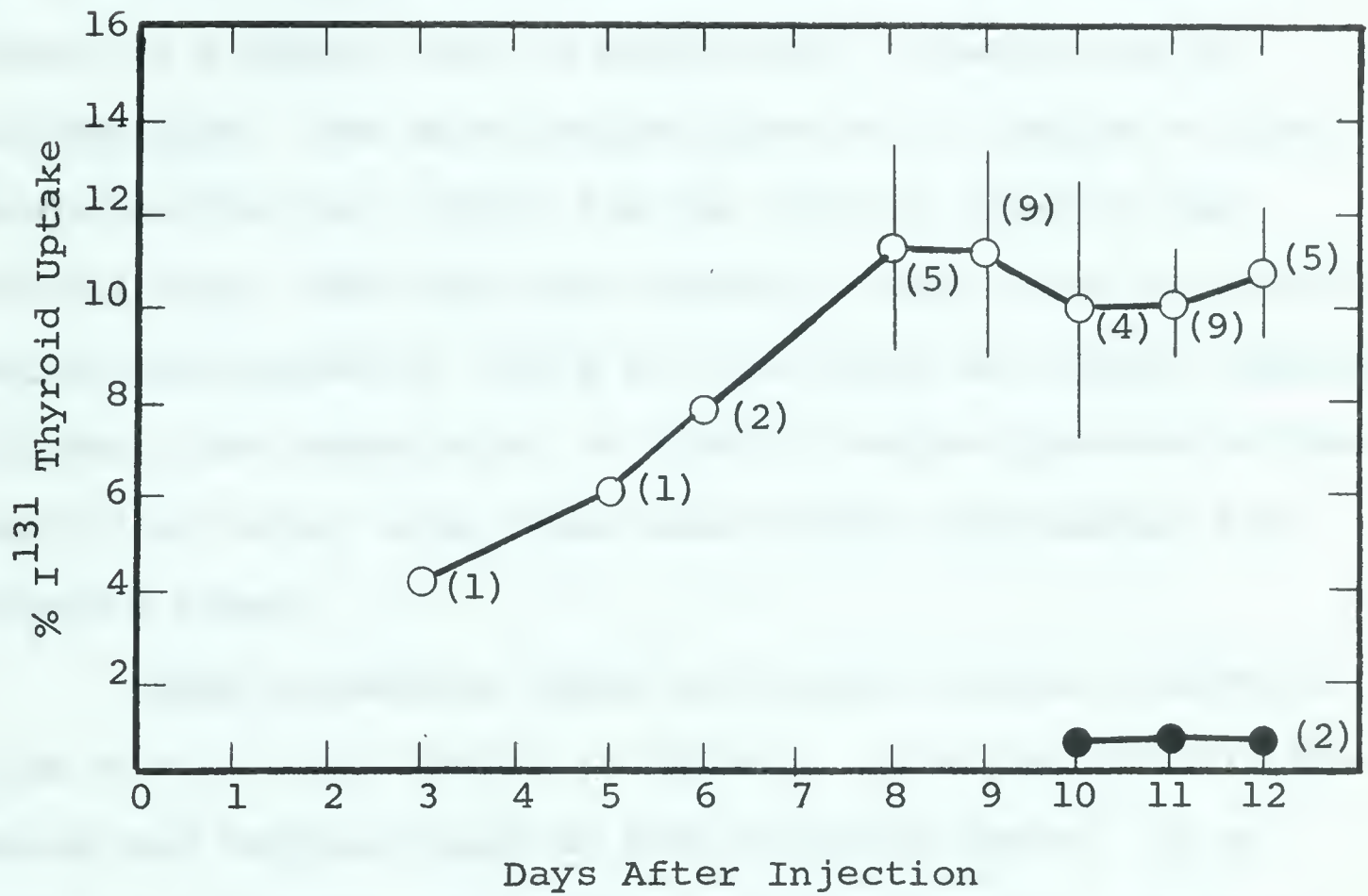


Fig. 6.  $I^{131}$  uptake of normal (open circles) and propylthiouracil inhibited (closed circles) winter frogs. Numbers to the right of the points indicate number of samples.



thyroid gland of normal and PTU inhibited frogs were studied by radioautography. Since the fixing process is known to wash out any inorganic  $I^{131}$  present (Ford, 1957; Matthews, 1950), the darkening of the emulsion was presumably due to organic  $I^{131}$ . Plate 3 (APPENDIX) shows the appearance and concentration of  $I^{131}$  by the thyroid gland of a normal frog 18 hours after injection of 10 microcuries. The histological picture is similar to that described by Joel (1949) for the thyroid gland of the winter frog. The follicles contain a great deal of colloid, while the epithelial cells are flattened and almost squamous. Although not shown here, no organic iodine appeared in the cartilage cells, even those immediately surrounding the thyroid gland.

Both conversion ratio and uptake studies indicated that a very small amount of organic iodine appeared in the blood and thyroid gland of PTU inhibited frogs. In an attempt to locate this iodine, radioautographs were prepared from the thyroids of a normal and a PTU inhibited frog removed two days after injection of  $I^{131}$ . It is evident that the thyroid of the PTU-fed frog (Plate 4) contained very little  $I^{131}$ , which was sparsely scattered throughout the colloid. By comparison, the radioautograph of the normal frog was completely darkened by radiation and could not be photographed.





and thiourea failed to induce any histological change in the thyroid gland of adult Rana nigromaculata nigromaculata, although it should be mentioned that histological appearance is not the most sensitive or accurate criterion of thyroid activity.

These findings contrast with those of other investigators who have found that stimulation or inhibition of thyroid function resulted in changes in thyroid activity. Surgical removal of both thyroids lowered the metabolic rate of salamanders while the implantation of 4 extra thyroids increased it (Taylor, 1936, 1939). Warren (1940) has shown that injections of thyroxine and thyroid glands over twelve weeks will raise the oxygen consumption of winter frogs. Calhoun (1955) observed a 26% decrease in the metabolic rate of summer frogs measured at 30°C after one week of treatment with PTU. By the fifth and last week of the experiment the metabolic rate had decreased to 40% below the control frogs. However, these frogs were not fed, and it has been observed that starvation suppresses oxidative metabolism in frogs (Joel, 1949; Warren, 1940).

It appears from my results and others that thyroid function is significantly affected by season. The seasonally cyclic nature of the amphibian thyroid has





## DISCUSSION

### Factors Affecting Metabolic Rate

It is universally accepted that the thyroid gland of homiotherms is intimately associated with and responsible for the regulation of respiratory metabolism. This association is less discernible in the amphibian, whose metabolic rate fluctuates with the season and environmental conditions. This study has shown that chemical inhibition of the thyroid gland with PTU for nine weeks did not significantly alter the resting metabolism of the winter frog. This suggests that the calorogenic action of the thyroid gland is repressed during the winter. Previous studies with amphibians give support to this view. Donoso (1958a), working with summer and winter toads, <sup>(*Bufo arenarum*)</sup> showed that a single injection of either thyroxine or triiodothyronine raised the oxygen consumption of adult toads considerably above normal for nine days. However, he observed no effect of hypophysectomy on the oxygen consumption and concluded that the thyroid gland is metabolically inactive in the adult. Joel (1949) failed to induce any histological changes in the thyroid of the winter frog until 4-7 weeks on thiouracil. More recently, Iwasawa (1959) has shown that injection of frog hypophyses



been well documented (Sklower, 1925; Smith, 1951), but there is yet a lack of quantitative information on the physiological processes involved. The frog thyroid appears to be inactive from November to February, active from April through August, and gradually declining in activity until the winter level is reached. This seasonal pattern has also been shown to exist in turtles (Claire, 1956) and in many species of fish (Swift, 1960).

By the use of the conversion ratio, this study has shown that there is a 50% reduction in the percentage of blood organic  $I^{131}$  from August to December, and a similar increase from December to February. Furthermore, the level of blood PBI $^{131}$  in December is only slightly higher than that present in PTU inhibited frogs. The raised blood PBI $^{131}$  level in February appears to compensate for the lower thyroid PBI level at this time. Thyroidal  $I^{127}$  estimates revealed that the gland contains one third the amount of PBI on February 23rd as on December 1st. This implies that thyroid hormone is being stored during the early winter and released into the bloodstream sometime in February, resulting in the higher serum PBI $^{131}$  level.

Seasonal variation in thyroxine secretion may be due to a change in the iodide binding capacity of the thyroid gland. The  $I^{131}$  uptake of turtles (Claire, 1956)





and fish (Matty, 1960) has been shown to follow a seasonal pattern, and there is evidence from the present study that thyroid  $I^{131}$  uptake of Rana pipiens increases towards the end of February. This implies that the thyroid has a different capacity for iodine at certain months of the year, which may be due to changes in thyroid stimulating hormone (TSH) levels. Lynn (1961) has shown that TSH causes a marked increase in  $I^{131}$  uptake by the normally inactive thyroid gland of Triturus. These results imply that the thyroid gland and tissues are always receptive to stimulation, but are normally inactive in the winter due to the decreased level of TSH.

The cyclic activity of the thyroid gland and other organs in the frog seem to be mainly governed by an intrinsic factor (van Oordt, 1960), but temperature also plays an important part. A high temperature will stimulate thyroid activity and increase the peripheral utilization of the thyroid hormone. This may be the cause of the sudden decrease in oxygen consumption shown by Calhoun (1955) in PTU injected frogs measured at 30°C. There appears to be a temperature below which the thyroid may be inactive (Shellabarger, 1956). Studies by Delsol (1956) and Warren (1940) in the frog have shown that the action of TSH and thyroxine are blocked below 12°C.



The fact that the holding temperature in this experiment remained between 13-15°C gave reasonable assurance that the winter thyroidal inactivity was not caused by low temperature acting to block thyroxine production.

### Thyroid Iodine Metabolism

#### A. Influence of Season

In assessing thyroid function, the most logical and seemingly accurate method would be to measure the amount of circulating thyroxine iodine (measured as protein-bound iodine) available for peripheral utilization. Until recently, this was thought to represent an accurate index of thyroid activity. Rigorous clinical studies had shown that the effect of goitrogens appear first as a drop in blood protein-bound iodine, followed by a decrease in the oxygen consumption (Curtis, 1948; Barker, 1949). However, recent observations have cast doubt on its reliability as an indicator of thyroid activity (Johnson, et al., 1959; Robertson, 1961). Robertson concluded that the protein-bound iodine level showed only a marginal correlation to the rate of thyroxine secretion. Leloup (1949) found that more active teleosts had a higher blood protein-bound iodine, but this was refuted by Matty (1960) who showed that an active marine fish (Scarus guacamaia)





had a low blood protein-bound iodine. Mellen (1957) found that blood protein-bound iodine was a poor indicator of thyroid activity in thiouracil treated fowl. No blood protein-bound iodine values were determined here for PTU inhibited frogs, but the low level in normal frogs (0.24 micrograms %), compared to 5.1 micrograms % for man (Barker, 1951) and 3.5 micrograms % in the rabbit (Mellen, 1957), demonstrates the minute quantity available for peripheral utilization. In view of the doubtful discriminatory value of blood protein-bound iodine, together with its low concentration in frog blood, it offers little as a worthwhile parameter of thyroid function in frogs.

Radioiodine proved to be a more useful indicator of thyroid activity, and was employed in this study to demonstrate the reduced iodine concentrating and secreting capacity of the winter thyroid gland. The low affinity of the thyroid for iodine was shown by the  $I^{131}$  uptake of 11.3% at the eighth day after injection. This is similar to Money (1955) who has shown that the uptake of  $I^{131}$  in the tadpole<sup>(*Rana pipiens*)</sup> reaches about 10-20% of an injected dose in ten days. However, it is much higher than the  $I^{131}$  uptakes reported by others for ~~the~~ adult frogs (Matthews, 1950; Volpert, 1958; Leloup, 1959) and the





toad (Bufo arenarum) (Donoso, 1958b). This may be due to the lower holding temperature (13-15°C) or simply that the uptake was followed over a longer period of time. The slow rate of iodine binding by the thyroid gland may be an important factor in the inactivity of the gland in adult Rana pipiens.

Conversion ratio studies showed that ten days elapsed before a maximum protein-bound iodine concentration of 7.5% appeared in the plasma. This is similar to the rainbow trout (Salmo gairdneri), which requires eight days before a conversion ratio of 3.0% is reached (Fontaine, 1959b). On the other hand, rats attain a conversion ratio of 58.5% at the 16th hour after injection (Chaikoff, 1947), while the normal conversion ratio of man ranges from 13-42% in 24 hours (Clark, 1949). This emphasizes the faster secretion rate of a unit dose of iodine by the more active glands of homoiothermic animals.

The eight day lapse before thyroid  $I^{131}$  equilibrium ( $I^{131}$  entering gland equals amount leaving) is reached and a further delay of two days before blood  $PBI^{131}$  equilibrium is attained demonstrates the slow iodine turnover rate of the frog thyroid. The combination of a slow output of thyroxine and triiodothyronine from the thyroid gland, which results in low circulating



levels of PBI may explain the failure of the thyroid gland to affect the oxygen consumption in the winter frog.

#### B. Effect of Propylthiouracil

Although propylthiouracil had no noticeable effect on the oxygen consumption,  $I^{131}$  studies showed a nearly complete blockage of iodine binding and secretion by the thyroid gland. The uptake of  $I^{131}$  by PTU treated frogs was decreased from 11.3% to 0.6% at the ninth week of the experiment. This is comparable to the findings of Matthews (1950) who reported a 0.29% uptake in thiouracil treated frogs compared to a normal uptake of 3.66%. The small amount of radioiodine taken up by the PTU blocked thyroid glands was found by radioautography to be scattered throughout the colloid (Plate 4, APPENDIX), demonstrating that a little organification took place. This is probably insignificant, however, compared to the normal accumulation of  $I^{131}$  by the follicles (Plate 3, APPENDIX).

The drop in  $I^{131}$  uptake by the chemically inhibited gland may lead to the erroneous conclusion that nearly all thyroid organic iodine had disappeared by the termination of the experiment. Chemical iodine analysis showed that the PBI and BEI had decreased by only 50% after nine





weeks. If blockage was instantaneous (Bradley, 1951), the biological half-life for the secretion of stored thyroid hormone would be around 60 days for the winter frog thyroid. It appears from these findings that PTU effectively blocked the organification of iodine, but even after two months had not depleted the thyroid iodine stores. This indicates the presence of a greatly reduced secretion rate and low circulating hormone level in the winter frog, which prevented the effect of PTU from being registered peripherally. If this is so, it follows that the thyroid gland is not influencing the metabolism of the normal winter adult frog.

#### C. Significance of Circulating Iodotyrosines

Although it has been stated that chemical protein-bound iodine measurements offered little as a test for thyroid function, a comparison of the relative concentrations of the iodoamines in the thyroid gland and blood proved valuable in explaining the inactivity of the winter thyroid gland. The process of thyroid hormone synthesis is well known (Astwood, 1949; Nadler, 1955). The thyroid gland picks up circulating iodide and oxidizes it into moniodotyrosine, diiodotyrosine, thyroxine, and triiodothyronine. These radicals are stored as thyroglobulin in



the colloid and released by a protease. Thyroxine and triiodothyronine enter the circulation via a concentration gradient, but the iodotyrosines are deiodinated to iodide in the thyroid gland. The enzyme responsible for this is a thermolabile, microsomal deiodinase found primarily in the thyroid, but is also present in the liver and kidney in smaller amounts (Stanbury, 1958; 1960).

The proportion of iodotyrosines in the thyroid gland is quite high. Radioiodine studies have indicated that they compose about 66% of the rat thyroidal iodine (Mayberry, 1960; Pitt-Rivers, 1961), and about 80% of the iodine in the frog thyroid (Leloup, 1960; Donoso, 1958b). This study has shown by chemical analysis that a higher amount, between 90-95%, exists as monoiodotyrosine and diiodotyrosine.

In mammals, all iodotyrosines that are released from thyroglobulin are broken down to iodide by the thyroidal deiodinase, and none appear in the blood (Gross, 1955; Barker, 1955, Pitt-Rivers, 1961). However, the blood of fish has been shown to contain varying amounts of these lower analogs (Leloup, 1949; Hickman, unpublished). This study has found about 70% of the total blood protein-bound iodine of the frog consists





of iodotyrosines. This is evidence that the thyroid enzyme responsible for the breakdown of these lower analogs to iodide is relatively ineffective or is lacking, allowing iodinated tyrosines to enter the circulation. Stanbury (1960) has shown that patients with hypothyroidism or familial goiter are unable to deiodinate iodotyrosines, and these compounds appear in the blood. Pitt-Rivers and Tata (1959) have reported the presence of a deiodinase in marine fish, but its functional value is not known.

It is suggested, as a possible explanation why these lower analogs are present in the blood, that the thyroid deiodinase is unable to function effectively or is missing entirely. There is evidence that a possible correlation exists between the presence of iodotyrosines in the circulation and the degree of thyroid activity. Those animals with iodotyrosines appearing in the blood also show a reduced thyroid function.





## CONCLUSIONS AND SUMMARY

Thyroid function in the adult winter frog was evaluated through a study of oxygen consumption and radioiodine metabolism. Chemical thyroidectomy with propylthiouracil had no effect on the respiratory metabolism of the winter frog. Since oxygen consumption is dependent upon the peripheral utilization of thyroid hormone, it appears that the thyroid gland is metabolically inactive during the winter.

Iodine metabolism studies confirmed the existence of seasonal changes in thyroid activity in the adult frog. The thyroid in December is characterized by iodine storage and in late February by iodine release. From December 1 to February 23, thyroid protein-bound iodine was reduced from 4.3 to 1.8 micrograms, while blood protein-bound radioiodine rose from 3.2 to 6.8%. The December conversion ratio of 3.2% was only slightly above the conversion ratio of the propylthiouracil-fed frogs (1.5%), exemplifying the low secretion rate during the winter. Further evidence for this low secretion came from chemical iodine analyses, which showed that after two months of treatment, propylthiouracil blocked thyroids had lost only one-half their pre-treatment concentration of organic iodine.



Season was found to influence the capacity of the thyroid for iodine, but not the iodine turnover rates. Radioiodine studies showed a constant thyroid  $I^{131}$  binding and secretion rate of 8 and 10 days, respectively. By contrast, the concentration of iodine in the blood and thyroid gland has been shown to vary with the season. It appears that the differential capacity of the thyroid gland for iodine is responsible for the cyclic nature of thyroid activity. This affinity for iodine has been thought to be governed by variations in TSH level. The winter thyroid, having only a token capacity to bind circulating iodide, is wholly unable to regulate the oxidative metabolism.

This study has confirmed the abundance of iodotyrosines in the thyroid gland, and has also demonstrated their subsequent release into the circulation. Tyrosine radicals are present in the blood of fishes, but in mammals they are normally absent, appearing only in cases of human familial goiter and hypothyroidism. It is proposed that thyroidal deiodinase, which normally reduces iodotyrosines to iodide, is not functioning effectively in poikilotherms, allowing iodinated tyrosines to appear in the blood. Thus the normal presence of these iodotyrosines in the circulation is associated with the low thyroid activity in frogs and other poikilotherms.





All available evidence suggests that the thyroid gland, between late summer and early winter, regresses to a point where it no longer influences the respiratory metabolism of the frog. The higher thyroid activity in August and February raises the possibility of a more calorìgenically important gland at other seasons. Until a study is performed in the summer frog under similar conditions of temperature and feeding, the metabolic significance of the adult amphibian thyroid gland must remain questionable.



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## APPENDIX





Table III

EFFECT OF PROPYLTHIOURACIL ON OXYGEN CONSUMPTION									
Propylthiouracil Frogs									
Frog	Week								
	1	2	3	4	5	6	7	8	9
1	83.5	93.1	118.4	79.3	105.8	111.4	56.2	45.3	38.5
2	45.7	75.0	52.8	45.9	60.3	51.7	62.1	45.2	43.9
3	60.7	87.9	95.3	103.6	57.0	50.3	51.6	36.4	47.1
4	52.5	42.0	47.5	43.7	44.8	45.7	42.9	38.8	36.0
5	60.0	81.7	58.0	54.8	51.3	47.5	46.3	49.0	45.5
6	52.8	53.4	45.0	42.6	50.4	48.7	45.9	41.6	36.1
7	85.6	97.4	100.6	66.4	64.2	51.0	42.9	40.1	38.6
8	54.7	41.9	46.6	46.4	51.2	65.5	53.5	48.5	50.1
9	53.3	65.0	67.2	77.4	63.3	60.1	54.1	47.0	45.9
Mean $\bar{x}$	61.0	68.7	67.0	62.2	60.9	59.1	50.6	43.5	42.4
%Change	+4.8	+18.0	+15.1	+6.9	+4.6	+1.5	-13.1	-25.3	-27.2
Control Frogs									
Frog	Week								
	1	2	3	4	5	6	7	8	9
1	72.1	53.7	57.4	65.9	58.5	52.1	48.5	51.0	37.2
2	78.0	58.1	61.2	52.6	65.7	90.8	46.7	47.5	39.8
3	69.3	91.6	80.1	87.9	124.3	54.4	63.0	44.2	54.2
4	84.8	52.9	117.0	87.3	68.1	113.7	47.5	50.3	51.9
5	39.1	55.8	63.1	43.8	57.7	47.1	39.5	41.9	38.2
6	54.0	57.8	85.6	60.8	66.5	66.6	56.6	49.4	45.1
7	47.5	53.3	59.2	46.5	46.7	57.7	38.0	36.4	30.1
8	66.5	59.8	66.2	76.8	54.0	40.9	38.0	52.8	38.9
9	62.9	63.4	55.4	67.8	51.4	63.4	40.9	43.5	34.1
Mean $\bar{x}$	63.8	60.7	71.7	65.5	65.9	65.2	46.5	46.3	41.0
%Change	+15.4	+9.8	+29.6	+18.4	+19.2	+17.9	-15.9	-16.3	-25.9



Table IV

EFFECT OF PROPYLTHIOURACIL ON BODY WEIGHT

<u>Propylthiouracil Frogs</u>									
<u>Frog</u>	<u>Week</u>								
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>	<u>9</u>
1	42.5	41.9	41.4	41.1	41.6	40.4	40.2	41.2	42.9
2	41.6	41.8	43.6	42.1	42.0	40.6	41.6	40.8	41.3
3	46.9	46.5	46.4	46.7	46.8	47.0	46.5	50.4	46.7
4	45.7	46.0	46.3	45.8	45.2	45.7	45.1	45.5	44.4
5	45.0	42.4	45.7	45.0	46.8	48.4	49.7	49.0	49.8
6	39.1	39.9	40.0	45.8	41.7	42.1	43.0	42.4	41.4
7	46.7	46.2	44.2	45.2	45.2	45.1	46.6	46.5	46.1
8	51.8	50.1	49.4	49.6	44.9	44.3	43.9	45.0	44.8
9	40.0	40.5	39.2	38.1	39.5	40.7	41.3	41.0	40.3
Mean $\bar{x}$	44.0	43.6	43.7	44.4	43.7	43.8	44.2	44.6	44.2
%Change		-2.4	-4.1	-2.9	-4.1	-5.8	-5.3	-6.0	-5.0

<u>Control Frogs</u>									
<u>Frog</u>	<u>Week</u>								
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>	<u>9</u>
1	43.9	42.2	41.8	43.0	41.9	40.9	40.7	39.2	40.3
2	35.9	35.0	34.3	35.5	35.0	34.6	36.4	36.3	36.8
3	41.1	40.4	38.7	40.4	37.0	37.4	34.4	36.9	36.9
4	38.3	37.8	35.9	37.8	36.1	34.0	36.5	35.8	35.3
5	45.2	43.0	42.8	43.4	41.6	42.5	41.6	40.0	40.6
6	42.0	41.5	39.7	39.5	39.1	38.0	38.0	38.5	38.8
7	42.1	41.3	42.2	41.6	42.8	41.6	42.1	41.2	42.1
8	43.1	42.8	42.3	42.1	43.3	43.0	43.8	42.6	42.8
9	43.4	42.6	42.1	41.3	42.8	41.5	42.2	42.1	43.0
Mean $\bar{x}$	41.7	40.7	40.0	40.5	40.0	39.3	39.5	39.2	39.6
%Change		-0.9	-0.7	+0.9	-0.7	-0.5	+0.5	+1.4	-0.4





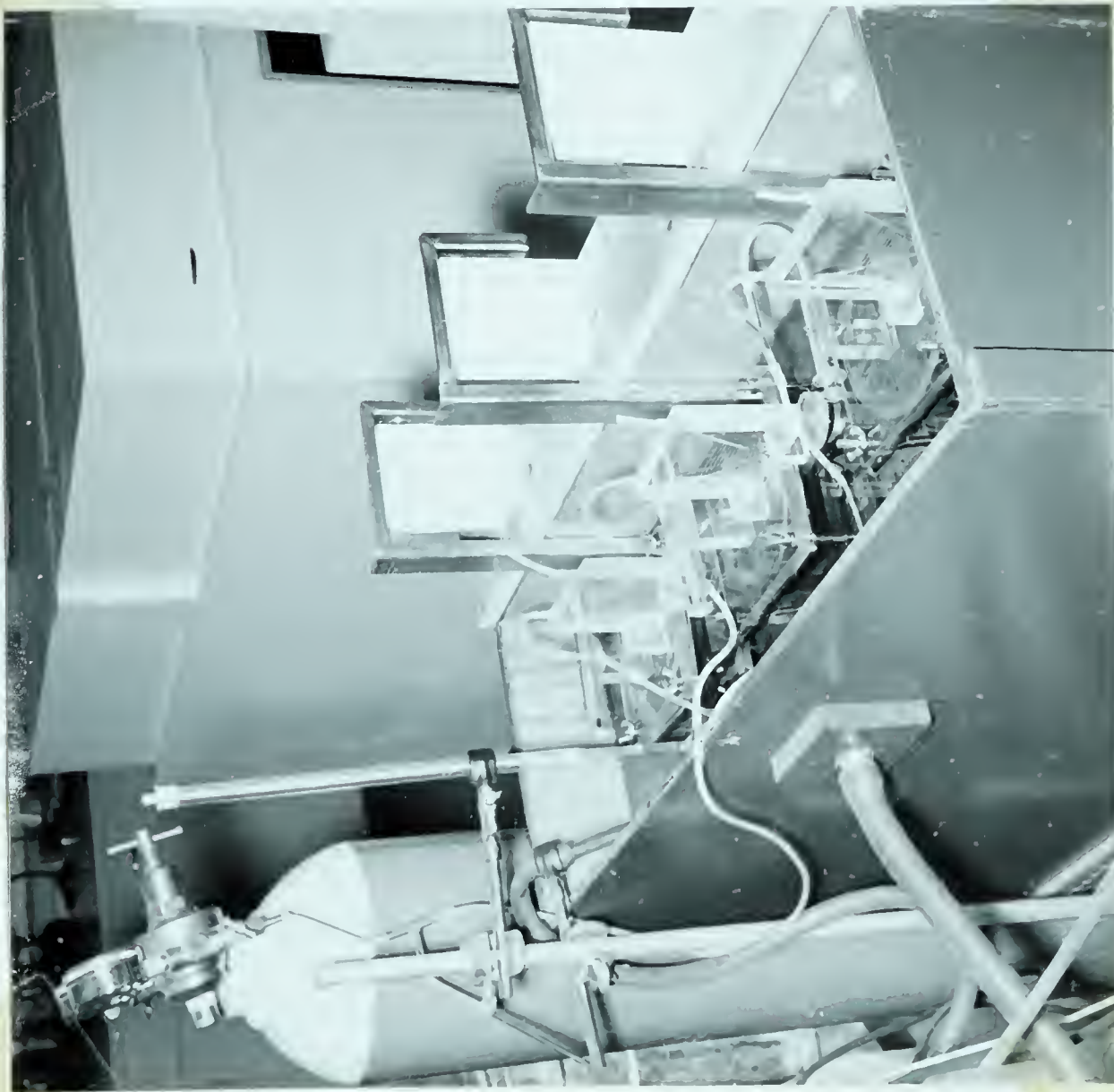


Plate 1. Respirometers partially submerged - night position.

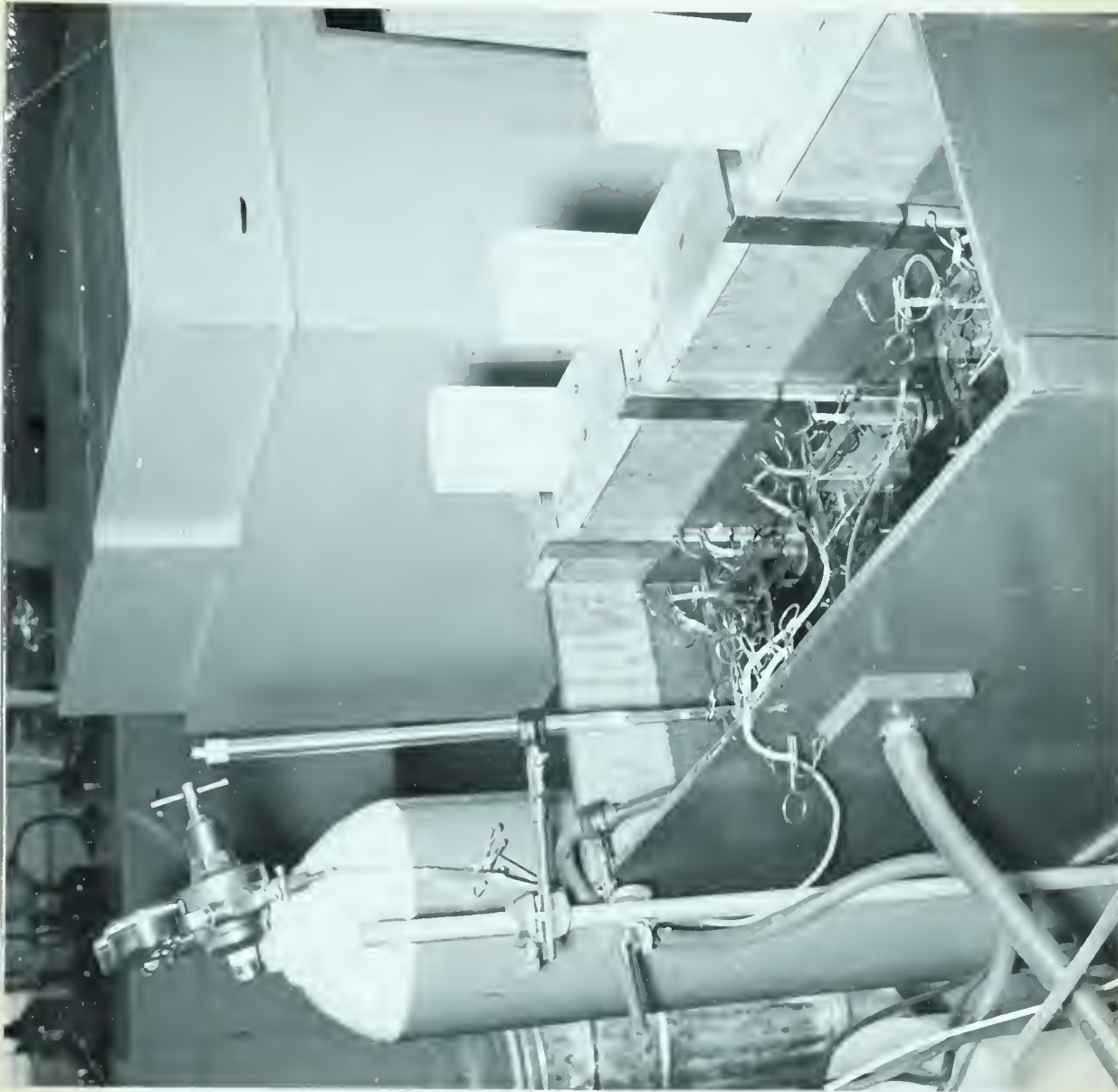


Plate 2. Respirometers totally submerged - measuring position.





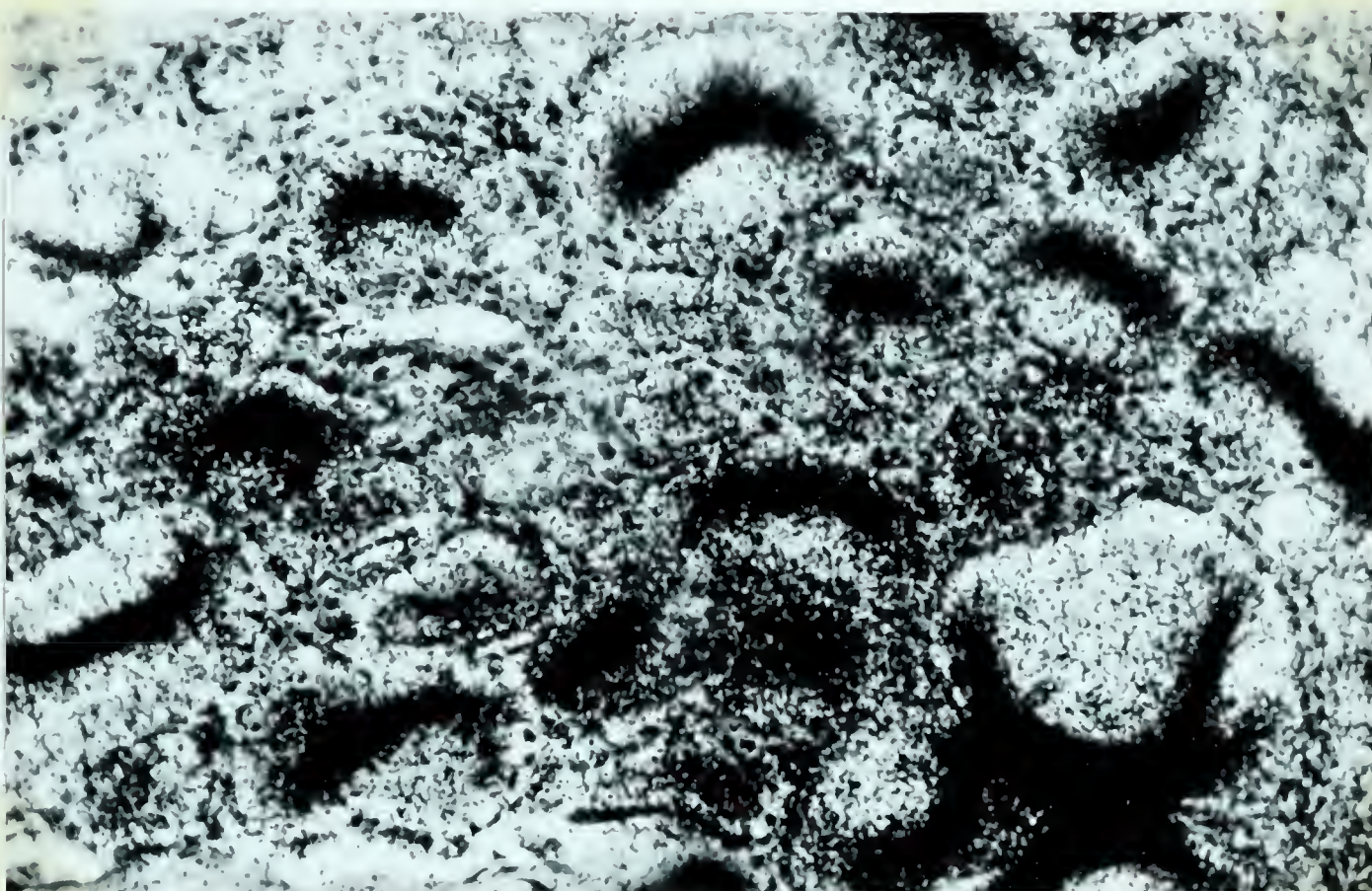


Plate 3. Radioautograph of thyroid of normal frog, killed 18 hours after receiving 10  $\mu$ c  $I^{131}$ . x200

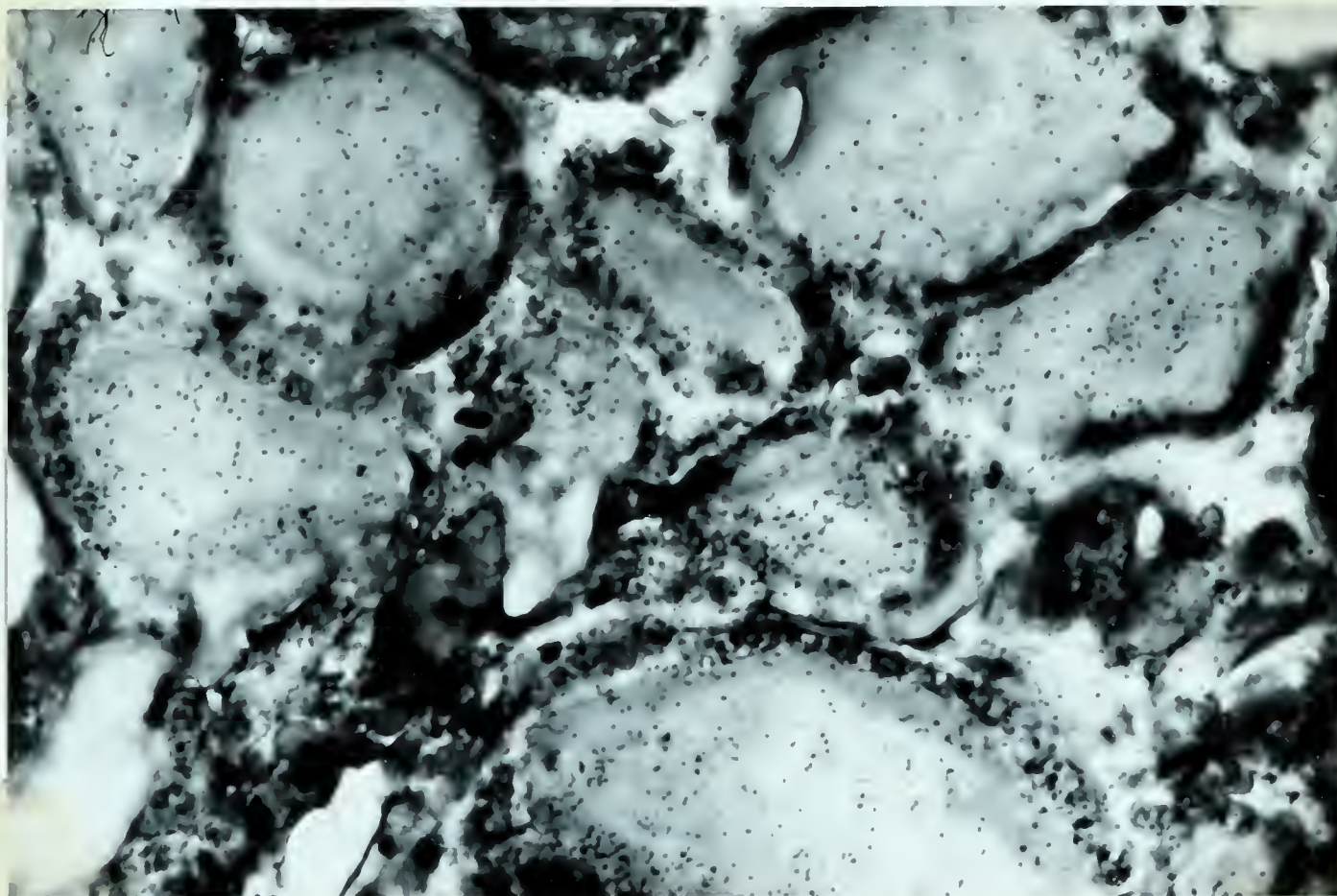


Plate 4. Radioautograph of thyroid of propylthiouracil inhibited frog, killed 48 hours after receiving 10  $\mu$ c  $I^{131}$ . x350













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